UTILITY PATENT APPLICATION TRANSMITTAL

(Only for new nonprovisional applications under 37 CFR 1 53(b))

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First Inventor or Application Identifier	Vitaliy A. LIVSHITS, et al.
Title	METHOD FOR PRODUCING L-AMINO ACID

APPLICATION ELEMENTS

See MPEP chapter 600 concerning utility patent application contents

ADDRESS TO: Assistant Commissioner for Patents
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1. ■ Fee Transmittal Form (e.g. PTO/SB/17)
(Submit an original and a duplicate for fee processing)

2. ■ Specification — Total Pages: 58

3. □ Drawing(s) *(35 U.S.C. 113)* — Total Sheets:

4. ■ Oath or Declaration — Total Pages: 4
   a. ■ Newly executed (original)
   b. □ Copy from a prior application *(37 C.F.R. §1.63(d))*
   (for continuation/divisional with box 15 completed)
   i. □

DELETION OF INVENTOR(S)
Signed statement attached deleting inventor(s) named in the prior application, see *37 C.F.R. §1.63(d)(2) and 1.33(b).*

5. □ Incorporation By Reference *(usable if box 4B is checked)*
The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied under Box 4B, is considered to be part of the disclosure of the accompanying application and is hereby incorporated by reference therein.

ACCOMPANYING APPLICATION PARTS

6. □ Assignment Papers (cover sheet & document(s))
7. □ 37 C.F.R. §3.73(b) Statement *(when there is an assignee)* □ Power of Attorney
8. □ English Translation Document *(if applicable)*
9. □ Information Disclosure Statement (IDS)/PTO-1449 □ Copies of IDS Citations
10. □ Preliminary Amendment
11. ■ White Advance Serial No. Postcard
12. □ Small Entity Statement(s) □ Statement filed in prior application. Status still proper and desired.
13. ■ Certified Copy of Priority Document(s)(2)
(if foreign priority is claimed)
14. ■ Other: Notice of Priority, Receipt of an Original Deposit of Microorganisms for the Purposes of Patent Procedure (VKPM B-7707, VKPM B-7708, VKPM B-7712, VKPM B-7713, VKPM B-7714, VKPM B-7715, VKPM B-7716, VKPM B-7718, VKPM B-7719, VKPM B-7722, VKPM B-7728, VKPM B-7729 VKPM B-7730, VKPM B-7731 VKPM B-7748, VKPM B-7752 VKPM B-7753, VKPM B-7754)

15. If a CONTINUING APPLICATION, *check appropriate box, and supply the requisite information below:*
□ Continuation □ Divisional □ Continuation-in-part (CIP) of prior application no.:
Prior application information: Examiner: Group Art Unit:

16. Amend the specification by inserting before the first line the sentence:
□ This application is a □ Continuation □ Division □ Continuation-in-part (CIP)
of application Serial No. Filed on
□ This application claims priority of provisional application Serial No. Filed

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METHOD FOR PRODUCING L-AMINO ACID

Technical Field

The present invention relates to a method for producing an amino acid. In particular, the present invention relates to an L-amino acid-producing bacterium belonging to the genus *Escherichia* and a method for producing L-amino acids, more specifically, L-glutamic acid, L-lysine, L-threonine, L-alanine, L-histidine, L-proline, L-arginine, L-valine, and L-isoleucine, using the bacterium.

Background Art

For production of an L-amino acid by fermentation, a strain isolated from the natural world or an artificial mutant of the strain has been used to improve productivity. For example, in the case of L-lysine, many artificial mutants producing L-lysine are known, and most of them are mutants resistant to S-2-aminoethylcysteine (AEC) and belong to the genus *Brevibacterium*, *Corynebacterium*, *Bacillus* or *Escherichia*. Also, there have been proposed various technics for increasing amino acid production such as use of a transformant obtained by using a recombinant DNA (U.S. Patent No. 4,278,765).

The technics are mostly based on enhancement of an activity of an enzyme involved in an amino acid biosynthetic pathway, conversion of the enzyme to that desensitized in

inhibition and the like (As to bacterium belonging the genus *Escherichia*, see Japanese Patent Application Laid-Open No. 56-18596 (1981) and International Publication No. WO 95/16042).

On the other hand, as an example of improvement of amino acid productivity by enhancing an amino acid excretion protein, a bacterium belonging to the genus *Corynebacterium* in which an L-lysine excretion gene, *lysE* is enhanced is known. However, as to bacteria belonging to the genus *Escherichia*, it is unknown even whether an L-amino acid excretion protein is present or not. Therefore, it is unknown whether enhancement of the L-amino acid excretion protein is effective in L-amino acid production using a bacterium belonging to the genus *Escherichia* or not.

Although the entire nucleotide sequence of *E. coli* strain K-12 belonging to the genus *Escherichia* has been already determined (Science, 277, 1453-1474(1997)), there are a large number of proteins of which functions are unknown.

Disclosure of the Invention

An object of the present invention is to obtain a protein participating in excretion of an L-amino acid, thereby providing a strain improved in L-amino acid productivity and an improved method for producing an L-amino acid by fermentation.

The inventors have conducted screening for the protein

participating in excretion of an L-amino acid. As a result, the present inventors have found that a yield of an L-amino acid based on consumed sugar is increased when a particular gene is enhanced. On the basis of the finding, the present invention has been completed.

Thus, the present invention provides a bacterium belonging to the genus *Escherichia* and having an ability to produce an L-amino acid, wherein the ability to produce the L-amino acid is increased by increasing an expression amount of at least one protein selected from the group consisting of the following proteins of (A) to (H) (hereinafter also referred to as "the bacterium of the present invention"):

(A) a protein having an amino acid sequence shown in SEQ ID NO: 10 in Sequence Listing;

(B) a protein which has an amino acid sequence including deletion, substitution, insertion, addition or inversion of one or several amino acids in the amino acid sequence shown in SEQ ID NO: 10 in Sequence Listing, and which has an activity of increasing the ability to produce the L-amino acid of the bacterium having the protein;

(C) a protein having an amino acid sequence shown in SEQ ID NO: 12 in Sequence Listing;

(D) a protein which has an amino acid sequence including deletion, substitution, insertion, addition or inversion of one or several amino acids in the amino acid sequence shown in SEQ ID NO: 12 in Sequence Listing, and which has an activity of increasing the ability to produce the

L-amino acid of the bacterium having the protein;

(E) a protein having an amino acid sequence shown in SEQ ID NO: 14 in Sequence Listing;

(F) a protein which has an amino acid sequence including deletion, substitution, insertion, addition or inversion of one or several amino acids in the amino acid sequence shown in SEQ ID NO: 14 in Sequence Listing, and which has an activity of increasing the ability to produce the L-amino acid of the bacterium having the protein;

(G) a protein having an amino acid sequence shown in SEQ ID NO: 16 in Sequence Listing; or

(H) a protein which has an amino acid sequence including deletion, substitution, insertion, addition or inversion of one or several amino acids in the amino acid sequence shown in SEQ ID NO: 16 in Sequence Listing, and which has an activity of increasing the ability to produce the L-amino acid of the bacterium having the protein.

The bacterium of the present invention preferably an L-lysine-producing bacterium in which an expression amount of at least one protein selected from the group consisting of the proteins (A) to (D), (G) and (H) is increased; an L-glutamic acid-producing bacterium in which an expression amount of at least one protein selected from the group consisting of the proteins (A) to (H) is increased; an L-alanine-producing bacterium in which an expression amount of at least one protein selected from the group consisting of the proteins (C) and (D) is increased; an L-valine-

producing bacterium in which an expression amount of at least one protein selected from the group consisting of the proteins (C) and (D) is increased; an L-histidine-producing bacterium in which an expression amount of at least one protein selected from the group consisting of said proteins (C) to (F) is increased; an L-proline-producing bacterium in which an expression amount of at least one protein selected from the group consisting of said proteins (A) to (F) is increased; an L-threonine-producing bacterium in which an expression amount of at least one protein selected from the group consisting of said proteins (E) and (F) is increased; an L-arginine-producing bacterium in which an expression amount of at least one protein selected from the group consisting of said proteins (G) and (H) is increased; or an L-isoleucine-producing bacterium in which an expression amount of at least one protein selected from the group consisting of said proteins (C) and (D) is increased.

Preferably, in the bacterium of the present invention, a copy number of a DNA coding for said protein in a cell is increased. The DNA is preferably carried on a multicopy vector in the cell or on a transposon in the cell.

The present invention also provides a method for producing an L-amino acid, comprising the steps of:

cultivating the bacterium of the present invention in a culture medium, to produce and accumulate the L-amino acid in the medium, and

recovering the L-amino acid from the medium

(hereinafter also referred to as "the bacterium of the present invention").

The method of the present invention preferably an L-lysine production method using an L-lysine-producing bacterium in which an expression amount of at least one protein selected from the group consisting of the proteins (A) to (D), (G) and (H) is increased; an L-glutamic acid production method using an L-glutamic acid-producing bacterium in which an expression amount of at least one protein selected from the group consisting of the proteins (A) to (H) is increased; an L-alanine production method using an L-alanine-producing bacterium in which an expression amount of at least one protein selected from the group consisting of the proteins (C) and (D) is increased; an L-valine production method using an L-valine-producing bacterium in which an expression amount of at least one protein selected from the group consisting of the proteins (C) and (D) is increased; an L-histidine production method using an L-histidine-producing bacterium in which an expression amount of at least one protein selected from the group consisting of said proteins (C) to (F) is increased; an L-proline production method using an L-proline-producing bacterium in which an expression amount of at least one protein selected from the group consisting of said proteins (A) to (F) is increased; an L-threonine production method using an L-threonine-producing bacterium in which an expression amount of at least one protein selected from the

group consisting of said proteins (E) and (F) is increased; an L-arginine production method using an L-arginine-producing bacterium in which an expression amount of at least one protein selected from the group consisting of said proteins (G) and (H) is increased; or an L-isoleucine production method using an L-isoleucine-producing bacterium in which an expression amount of at least one protein selected from the group consisting of said proteins (C) and (D) is increased.

Preferably, in the method of the present invention, a copy number of a DNA coding for said protein in a cell of the bacterium is increased. The DNA is preferably carried on a multicopy vector in the cell, or on a transposon in the cell.

According to the present invention, an ability to produce an L-amino acid of a bacterium belonging to the genus *Escherichia* can be increased. Also, a method for producing an L-amino acid can be improved in a production rate of an L-amino acid.

The present invention will be explained in detail below. Hereinafter, an amino acid is of L-configuration unless otherwise noted.

<1> Bacterium of the present invention

The bacterium of the present invention is a bacterium belonging to the genus *Escherichia* and having an ability to produce an amino acid, in which the ability to produce the amino acid is increased by increasing an expression amount

of a protein which has an activity of increasing the ability to produce the amino acid of the bacterium, or an activity of increasing resistance to an amino acid or amino acid analogue. Hereinafter, the protein is referred to as "amino acid excretion protein" for the sake of convenience. However, the term does not mean that function of the protein is limited to amino acid excretion.

Examples of the amino acid excretion protein include a protein having an amino acid sequence shown in SEQ ID NO: 10, a protein having an amino acid sequence shown in SEQ ID NO: 12, a protein having an amino acid sequence shown in SEQ ID NO: 14 and a protein having an amino acid sequence shown in SEQ ID NO: 16.

The amino acid excretion protein may have selectivity to amino acid. An amino acid excretion protein appropriate for each amino acid can be determined by allowing the amino acid excretion protein to be expressed in a bacterium belonging to the genus *Escherichia* and having an ability to produce the amino acid, and measuring an increase of a yield of the amino acid or measuring an increase of a minimum inhibition concentration (MIC) of an amino acid or amino acid analogue.

For example, in the case of lysine, a protein having an amino acid sequence shown in SEQ ID NO: 10, 12 or 16 is effective; in the case of glutamic acid, a protein having an amino acid sequence shown in SEQ ID NO: 10, 12, 14 or 16 is effective; in the case of alanine, a protein having an

amino acid sequence shown in SEQ ID NO: 12 is effective; in the case of valine, a protein having an amino acid sequence shown in SEQ ID NO: 12 is effective; in the case of histidine, a protein having an amino acid sequence shown in SEQ ID NO: 12 or 14; in the case of proline, a protein having an amino acid sequence shown in SEQ ID NO: 10, 12 or 14 is effective; in the case of threonine, a protein having an amino acid sequence shown in SEQ ID NO: 14 is effective; in the case of arginine, a protein having an amino acid sequence shown in SEQ ID NO: 16 is effective; and in the case of isoleucine, a protein having an amino acid sequence shown in SEQ ID NO: 12 is effective.

The term "an expression amount is increased" used herein usually means that the expression amount is larger than that in a wild strain of *E. coli* such as strain MG1655 or W3110. The terms also means that when a strain is obtained by modification through genetic engineering technics or the like, the expression amount is larger than that prior to the modification. The expression amount of the amino acid excretion protein may be determined directly by the determination of the amino acid excretion protein or indirectly by the determination of MIC of an amino acid or amino acid analogue or of amino acid productivity of a bacterium belonging to the genus *Escherichia* and having the amino acid excretion protein.

The method for increasing the expression amount of the amino acid excretion protein is exemplified by a method for

increasing a copy number of DNA encoding the amino acid excretion protein in a cell of the bacterium.

For increasing the copy number in the cell, a DNA fragment coding for the amino acid excretion protein may be ligated to a vector which functions in a bacterium belonging to the genus *Escherichia* to produce a recombinant DNA, which is introduced to a host to transform it. The copy number of the gene coding for the amino acid excretion protein (amino acid excretion protein gene) in the cell of the transformant strain increases, thereby increasing the expression amount of the amino acid excretion protein. The vector is preferably a multicopy vector.

The increase of the copy number in the cell can be achieved by allowing plural copies of the amino acid excretion protein gene to exist on chromosomal DNA of the host. The introduction of plural copies of the amino acid excretion protein gene to chromosomal DNA of a bacterium belonging to the genus *Escherichia*, may be conducted through homologous recombination by using a sequence of which plural copies exist on the chromosomal DNA, as a target. As the sequence of which plural copies exist on the chromosomal DNA, a repetitive DNA and an inverted repeat present in a terminal portion of a transposable element may be used.
Alternatively, as disclosed in Japanese Patent Application Laid-Open No. 2-109985 (1990), the plural copies can be introduced to the chromosomal DNA by making the amino acid excretion protein gene carried on a transposon and allowing

the transposon to be transposed, which is preferred. According to any of the above-mentioned methods, the copy number of the amino acid excretion protein gene in the transformant strain increases, thereby increasing the expression amount of the amino acid excretion protein.

The multicopy vector is exemplified by plasmid vectors such as pBR322, pMW118, pUC19 or the like, and phage vectors such as λ1059, λBF101, M13mp9 or the like. The transposon is exemplified by Mu, Tn10, Tn5 or the like.

The introduction of a DNA into a bacterium belonging to the genus *Escherichia* can be performed, for example, by a method of D. M. Morrison (Methods in Enzymology 68, 326 (1979)) or a method in which recipient bacterial cells are treated with calcium chloride to increase permeability of DNA (Mandel, M. and Higa, A., J. Mol. Biol., 53, 159 (1970)) and the like.

Besides the above-mentioned gene amplification, the increase of the expression amount of the amino acid excretion protein can be also achieved by replacing an expression regulatory sequence such as a promoter of the amino acid excretion protein gene with stronger one (see Japanese Patent Application Laid-Open No. 1-215280 (1989)). For example, *lac* promoter, *trp* promoter, *tac* promoter, P_R promoter and P_L promoter of lambda phage, and the like are known as a strong promoter. The replacement with the promoter enhances expression of the amino acid excretion protein, thereby increasing the expression amount of the

amino acid excretion protein. The enhancement of the expression regulatory sequence may be combined with the increase of the copy number of the amino acid excretion protein.

In the bacterium of the present invention, expression amounts of plural amino acid excretion proteins may be increased.

The amino acid excretion protein is encoded by genes which are known as *yahN* gene, *yeaS* gene, *yfiK* gene and *yggA* gene and of which functions are unknown. Therefore, the DNA encoding the amino acid excretion protein can be obtained by synthesizing primers based on the known sequences (for example, the entire nucleotide sequence of chromosome of *Escherichia coli* strain K-12 has been already determined (Science, 277, 1453-1474(1997))), and conducting amplification by PCR using chromosomal DNA of a bacterium belonging to the genus *Escherichia* as a template. Also, the object DNA fragment can be selected by hybridization from a chromosomal DNA library of a bacterium belonging to the genus *Escherichia* by preparing a probe based on the known sequences. Alternatively, the DNA encoding the amino acid excretion protein may be synthesized based on the known sequences. The nucleotide sequence of the DNA encoding the amino acid excretion protein is exemplified by that shown in SEQ ID NO: 9, 11, 13 or 15 in the Sequence Listing.

Methods for preparation of chromosomal DNA, preparation of chromosomal DNA library, hybridization, PCR,

preparation of plasmid DNA, digestion and ligation of DNA, transformation, selection of an oligonucleotide as a primer and the like may be ordinary methods well known to one skilled in the art. These methods are described in Sambrook, J., Fritsch, E. F., and Maniatis, T., "Molecular Cloning A Laboratory Manual, Second Edition", Cold Spring Harbor Laboratory Press (1989) and the like.

The amino acid excretion protein may comprise substitution, deletion, insertion, addition or inversion of one or several amino acids at one or a plurality of positions, provided that the activity of increasing the ability to produce the amino acid of the bacterium belonging to the genus *Escherichia* and having the protein is not deteriorated. The term "several" may vary depending on a position in a steric structure of the protein and a kind of an amino acid residue. It is because some amino acids such as isoleucine and valine have high similarity to each other, and a difference between such the amino acids does not largely affect the steric structure of the protein.

The DNA which codes for the substantially same protein as the amino acid excretion protein as described above, may be obtained, for example, by modifying the nucleotide sequence, for example, by means of the site-directed mutagenesis method so that one or more amino acid residues at a specified site involve substitution, deletion, insertion, addition or inversion. The DNA modified as described above may be obtained by the conventionally known

mutation treatment. The mutation treatment includes a method for treating a DNA coding for the amino acid excretion protein *in vitro*, for example, with hydroxylamine, and a method for treating a microorganism, for example, a bacterium belonging to the genus *Escherichia*, harboring a DNA coding for the amino acid excretion protein with ultraviolet irradiation or a mutating agent such as N-methyl-N'-nitro-N-nitrosoguanidine (NG) and nitrous acid usually used for the mutation treatment.

The substitution, deletion, insertion, addition or inversion of the one or more amino acid residues includes a naturally-occurring mutation or variation which is resulted from a difference between individual microorganisms having the amino acid excretion protein and a difference between species, strains or the like.

The DNA, which codes for substantially the same protein as the amino acid excretion protein, can be obtained by allowing a DNA having the mutation as described above to be expressed in a cell of an appropriate bacterium belonging to the genus *Escherichia*, and investigating the increase of amino acid productivity of the cell.

Also, the DNA, which codes for substantially the same protein as the amino acid excretion protein, can be obtained by isolating a DNA which hybridizes with DNA having, for example, a nucleotide sequence shown in SEQ ID NO: 9, 11, 13 or 15 in Sequence Listing under stringent conditions, and which codes for a protein having the activity of increasing

the ability to produce the amino acid of the bacterium belonging to the genus *Escherichia*, from DNAs encoding the amino acid excretion proteins having mutations or cells containing the DNAs. The term "stringent conditions" referred to herein means a condition under which a specific hybrid is formed, and a non-specific hybrid is not formed. It is difficult to clearly express this condition by using any numerical value. However, for example, the stringent conditions include a condition under which DNAs having high homology, for example, DNAs having homology of not less than 70% with each other are hybridized, and DNAs having homology lower than the above with each other are not hybridized, or a condition of a salt concentration corresponding to 60°C, 1x SSC, 0.1% SDS, preferably 0.1x SSC, 0.1% SDS which is a washing condition of ordinary Southern hybridization.

Although there may be a gene in which a stop codon is made in the middle, or a gene encoding a protein losing the activity due to mutation of the active center among the genes which hybridize under such the condition, such genes can be easily eliminated by ligating the genes to a commercially available activity-expression vector and determining the activity of increasing the ability to produce the amino acid of the bacterium belonging to the genus *Escherichia* as described above.

The term "DNA coding for a protein" used herein means a DNA of which one of strands codes for the protein when the DNA is double-stranded.

By increasing an expression amount of an amino acid excretion protein in an amino acid-producing bacterium belonging to the genus *Escherichia* as described above, a produced amount of the amino acid can be increased. As the bacterium belonging to the genus *Escherichia* in which the expression amount of the amino acid excretion protein is to be increased, strains which have abilities to produce desired amino acids (amino acid productivities) are used. Besides, an ability to produce an amino acid may be imparted to a bacterium in which the expression amount of the amino acid excretion protein is increased. Examples of amino acid-producing bacteria belonging to the genus *Escherichia* include *E. coli* AJ13199 (FR patent No. 2747689), and those obtainable from known materials (e.g., *E. coli* W3110 (tyrA)/pCABD2, *E. coli* VL614, *E. coli* VL2054, *E. coli* VL2160, *E. coli* VL2151, *E. coli* W3350 *argE*::Tn10/pKA10 as described in the Examples below).

For reference, the amino acid excretion protein according to the present invention was identified for the first time as described below.

The present inventors have identified *rhtB* and *rhtC* as threonine excretion protein genes of a bacterium belonging to the genus *Escherichia*. The present inventors searched databases based on a hypothesis that amino acid excretion proteins may share a common structure. Namely, BLAST and PSI-BLAST search (Altschul, S.F. et al., Nucleic Acids Res., 25, 3389-3402(1997)) for homology of a protein encoded by

rhtB was performed in GenBank CDS, PDB, SWISS-PROT, Spupdate and PIR. Tblastn search was performed in unfinished microbial genomes. BLITZ search (Sturrock, S.S., and Collins, J.F., Mpsch version 1.3. Biocomputing research unit University of Edinburgh, UK (1993)) was performed in SWALL database. SMART search (Ogiwara, I. et al., Protein Sci., 5, 1991-1999 (1996)) was performed in the databases of translations and SWISS-PROT. From the samples of more than 60 sequences found, YeaS (corresponding to f212 of ACCESSION No. AE000274 in GenBank), YahN (corresponding to f223 of ACCESSION No. AE000140 in GenBank), YfiK (corresponding to o195 of ACCESSION No. AE000344 in GenBank) and YggA (corresponding to f211 of ACCESSION No. AE000375 in GenBank) remained as proteins which may have similar function to RhtB, among those originating from *E. coli*. Since functions of any of these genes were unknown, the genes were actually obtained, and effects thereof on MIC of amino acids and amino acid analogues and on amino acid production were examined by enhancing activities thereof. As a result, an effect of increasing MIC of some amino acids and analogues was found with respect to YeaS, YfiK, YahN and YggA. Further examination has revealed that proteins encoded by these genes exhibit an effect of increasing an amino acid accumulation, although they may have some amino acid selectivities.

<2> Method of the present invention

The method of the present invention comprises the steps of cultivating the bacterium of the present invention, in a culture medium, to produce and accumulate the amino acid in the medium, and recovering the amino acid from the medium.

Suitable amino acids include lysine, glutamic acid, alanine, valine, homoserine, proline, and threonine.

In the method of present invention, the cultivation of the bacterium belonging to the genus *Escherichia*, the collection and purification of amino acid from the liquid medium may be performed in a manner similar to those of the conventional method for producing an amino acid by fermentation using a bacterium. A medium used in cultivation may be either a synthetic medium or a natural medium, so long as the medium includes a carbon and a nitrogen source and minerals and, if necessary, nutrients which the bacterium used requires for growth in appropriate amounts. The carbon source may include various carbohydrates such as glucose and sucrose, and various organic acids. Depending on assimilatory ability of the used bacterium, alcohol including ethanol and glycerol may be used. As the nitrogen source, ammonia, various ammonium salts such as ammonium sulfate, other nitrogen compounds such as amines, a natural nitrogen source such as peptone, soybean hydrolyte and digested fermentative microbe are used. As minerals, monopotassium phosphate, magnesium sulfate, sodium chloride, ferrous sulfate, manganese sulfate, calcium carbonate are used.

The cultivation is preferably culture under an aerobic condition such as a shaking culture, and an aeration and stirring culture. The temperature of culture is usually 20 to 40°C, preferably 30 to 38°C. The pH of the culture is usually between 5 and 9, preferably between 6.5 and 7.2. The pH of the culture can be adjusted with ammonia, calcium carbonate, various acids, various bases, and buffers. Usually, a 1 to 3-day cultivation leads to the accumulation of the target amino acid in the medium.

Recovering the amino acid can be performed by removing solids such as cells from the medium by centrifugation or membrane filtration after cultivation, and then collecting and purifying the target amino acid by ion exchange, concentration and crystalline fraction methods and the like.

Best Mode for Carrying Out the Invention

The present invention will be more concretely explained below with reference to Examples.

Example 1. Preparation of the DNA fragments which code for amino acid excretion proteins.

The entire nucleotide sequence of chromosome of *E. coli* strain K-12 has been determined (Science, 277, 1453-1474, 1997). Based on the reported nucleotide sequence, primers were synthesized and the genes *yahN*, *yfiK*, *yeaS* and *yggA* were amplified by PCR.

(1). Chromosomal DNA of the *E. coli* strain MG1655 was used as a template.

The chromosomal DNA was preapared by an ordinary method (Sambrook, J., Fritsch E. F. and Maniatis T. (1989) Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N. Y.). In the PCR reaction, a standard condition described in "PCR protocols. Current methods and applications". (White, B.A., ed. Humana Press, Totowa, New Jersey, 1993) was used. The obtained PCR products were purified by an ordinary method and digested with restriction enzymes as described below.

The *yahN* gene was amplified by using the primers No.1 and No. 2.

Primer No.1: gtgtggaaccgacgccggat (a sequence complementary to a sequence of from 1885 base to 1904 base in a nucleotide sequence registered under ACCESSION No. AE000140 in GenBank; SEQ ID NO: 17), and

Primer No.2: tgttgtatggtacggggttcgag (a sequence of from 223 base to 245 base in the same; SEQ ID NO: 18).

The obtained PCR product after purification was digested with restriction enzymes *Pst*I and *Stu*I and ligated to vector pUC21 (Vieira, Messing, Gene, 100, 189-194, 1991) digested with the enzymes *Pst*I and *Eco*RV by using a ligation kit. Then, transformation of competent cells of *E. coli* TG1 (Sambrook, J., Fritsch E. F. and Maniatis T. (1989) Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory

Press, Cold Spring Harbor, N. Y.) with the product was conducted and the cells were spread on L medium (10 g/l Bacto trypton, 5 g/l Yeast extract, 5 g/l NaCl, 15 g/l agar, pH 7.0) containing 10 mg/ml IPTG (isopropyl-β-D-thiogalactopyranoside) and 40 mg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) and 100 mg/ml ampicillin, and cultured overnight. Appeared white colonies were picked up and subjected to single colony isolation to obtain transformants. Plasmid was prepared from the transformants using an alkali extraction method and designated as pYAHN.

The *yeaS* gene was amplified by using the primers No.3 and No. 4.

Primer No.3: ctttgccaatcccgtctccc (a sequence complementary to a sequence of from 7683 base to 7702 base in a nucleotide sequence registered under ACCESSION No AE000274 in GenBank; SEQ ID NO: 19);

Primer No.4: gccccatgcataacggaaag (a sequence of from 5542 base to 5561 base in the same; SEQ ID NO: 19).

The obtained PCR product after purification was digested with a restriction enzyme *Ava*I and ligated to vector pUC19. After transformation of *E. coli* TG1 as above, the plasmid designated as pYEAS was obtained.

The *yfiK* gene was amplified by using the primers No.5 and No.6.

Primer No.5: gaagatcttgtaggccggataaggcg (a sequence of from 4155 base to 4177 base in a nucleotide sequence

registered under ACCESSION No AE000344 in GenBank, with a restriction enzyme *Bgl*II site added at the 5'-end thereof; SEQ ID NO: 21)

Primer No.6: tggttttaccaattggccgc (a sequence complementary to a sequence of from 6307 base to 6326 base in the same; SEQ ID NO: 22).

The obtained PCR product after purification was digested with restriction enzymes *Bgl*II and *Mun*I and ligated to vector pUC21 digested with restriction enzymes *Bgl*II and *Eco*RI. After transformation of *E. coli* TG1 as above, the plasmid designated pYFIK was obtained.

The *yggA* gene was amplified by using the primers No.7 and No.8.

Primer No.7: acttctcccgcgagccagttc (a sequence complementary to a sequence of from 9606 base to 9626 base in a nucleotide sequence registered under ACCESSION No AE000375 in GenBank; SEQ ID NO: 23).

Primer No.8: ggcaagcttagcgcctctgtt (a sequence of from 8478 base to 8498 base in the same; SEQ ID NO: 24).

The obtained PCR product after purification was digested with restriction enzymes *Hin*dIII and *Cla*I and ligated to vector pOK12 (Vieira, Messing, Gene, 100, 189-194, 1991) digested with the same restriction enzymes. After transformation of *E. coli* TG1 as above, the plasmid designated pYGGA was obtained.

(2). Chromosomal DNA of the *E. coli* strain W3110 was used as a template.

The *yahN* gene was amplified by using the primers No.9 (SEQ ID NO 1) and No. 10 (SEQ ID NO.2)

The *yeaS* gene was amplified by using the primers No.11 (SEQ ID NO 3) and No.12 (SEQ ID NO 4)

The *yfiK* gene was amplified by using the primers No.13 (SEQ ID NO 5) and No.14 (SEQ ID NO 6).

The *yggA* gene was amplified by using the primers No.15 (SEQ ID NO 7) and No.16 (SEQ ID NO 8)

The obtained PCR product was purified, digested with restriction enzymes *Sac*I and *Xba*I (*Eco*RI and *Pst*I for *yggA*), and ligated to plasmid pMW118 (Nippon Gene). The plasmid into which a DNA fragment of which sequence was identical to the reported sequence was inserted was designated as follows:

One carrying *yahN*: pMW118::*yahN*
One carrying *yeaS*: pMW118::*yeaS*
One carrying *yfiK*: pMW118::*yfiK*
One carrying *yggA*: pMW118::*yggA*

Example 2. Effect of the *yahN*, *yeaS*, *yfiK*, and *yggA* DNA fragments amplification on the *E. coli* TG1 resistance to some amino acids and amino acid analogues.

The homology of the *yeaS*, *yfiK*, *yahN* and *yggA* gene products with the lysine transporter, LysE, of

Corynebacterium glutamicum (Vrljic et al., Mol. Microbiol.,22, 815-826, 1996) and RhtB protein involved in homoserine excretion, indicates the analogues function for these proteins. It is well known that the increased expression of the genes involved in antibiotic and heavy metal efflux increases the level of resistance to the drugs (Nikaido, H. J. Bacteriology, 178, 5853-5859, 1996). Therefore, the effect of the pYEAS, pYAHN, pYFIK, and pYGGA plasmids on susceptibility of the strain TG1 to some amino acids and amino acid analogues was tested. Overnight cultures of the *E. coli* strains TG1/pYEAS, TG1/pYAHN, TG1/pYFIK, TG1/pYGGA and of the control strains TG1/pUC21, TG1/pUC19 and TG1/pOK12 grown in M9 minimal medium with an appropriate antibiotic on a rotary shaker (10^9 cfu/ml) were diluted 1:100 in M9 minimal medium and grown for 5 h in the same medium. Then the log phase cultures thus obtained were diluted and about 10^4 alive cells were applied to well-dried test plates with M9 agar containing doubling increments of amino acids or analogues. Thus the minimum inhibition concentration (MIC) of these compounds were examined.

The results are shown in Table 1. It follows from the Table 1 that multiple copies of *yfiK* gene conferred increased resistance to proline, homoserine, histidine, threonine, glutamate, lysine, α-amino-β-hydroxyvaleric-acid (AHVA), S-(2-aminoethyl)-L-cysteine (AEC) and α-aminobutyric acid; multiple copies of *yahN* gene conferred increased resistance to proline, multiple copies of *yeaS* gene conferred increased

resistance to threonine, homoserine, lysine, glutamate, histidine, proline and α-aminobutyric acid; multiple copies of *yggA* gene conferred increased resistance to S-(2-aminoethyl)-L-cysteine (AEC), lysine, and arginine. These results indicate that except for YahN, every of the presumed transporters have specificity to several substrates (amino acids and amino acid analogues), or may show non-specific effects as a result of amplification.

Table 1

Substrate	MIC (μg/ml) for *E. coli* TG1, harboring the plasmid				
	pUC21	pYFIK	pYAHN	pYEAS	pYGGA
L-homoserine	500	**1000**	500	**1000**	500
L-threonine	30000	**40000**	30000	**50000**	30000
L-lysine	5000	**7500**	5000	**7500**	**15000**
L-glutamate (Na salt)	5000	**10000**	5000	**20000**	5000
L-histidine	5000	**10000**	5000	**30000**	5000
L-valine	0.5	0.5	0.5	0.5	0.5
L-proline	1000	**5000**	**2000**	**2000**	1000
L-arginine	10000	10000	10000	10000	**20000**
AHVA	100	**200**	100	100	100
AEC	5	**10**	5	5	**200**
α-aminobutyric acid	2500	**5000**	2500	**>10000**	2500
4-aza-DL-leucine	100	100	100	100	100

Example 3. Effect of *yeaS*, *yahN*, and *yfiK* DNA fragments amplification on glutamic acid production.

The *E. coli* strain AJ13199 (FR patent No. 2747689) was transformed with the vector pUC21 and each of the plasmids

pYAHN, pYEAS and pYFIK. Thus the strains AJ13199/pUC21 (VKPM B-7728), AJ13199/pYAHN (VKPM B-7729), AJ13199/pYEAS (VKPM B-7731), and AJ13199/pYFIK (VKPM B-7730) were obtained.

These strains were each cultivated at 37°C for 18 hours in a nutrient broth with 100 mg/l ampicillin, and 0.3 ml of the obtained culture was inoculated into 3 ml of a fermentation medium containing 100 mg/l ampicillin, in a 20 x 200 mm test tube, and cultivated at 37°C for 48 hours with a rotary shaker. After the cultivation, an accumulated amount of glutamic acid in the medium was determined by known method.

The composition of the fermentation medium (g/l):

Glucose	80
$(NH_4)_2SO_4$	22
K_2HPO_4	2
NaCl	0.8
$MgSO_4 \cdot 7H_2O$	0.8
$FeSO_4 \cdot 7H_2O$	0.02
$MnSO_4 \cdot 5H_2O$	0.02
Thiamine HCl	0.0002
Yeast extract	1.0
$CaCO_3$	30.0 (dry-heat-sterilized at 180°C for 2 h)

(Glucose and K_2HPO_4 separately sterilized)

The results are shown in Table 2. As shown in Table 2, the strains AJ13199/pYAHN, AJ13199/pYEAS, and AJ13199/pYFIK accumulated glutamic acid in a larger amount than the strain AJ13199/pUC21 in which an expression amount of amino acid excretion proteins was not enhanced.

Table 2.

Strain	Glutamic acid, g/l
AJ13199/pUC21	21.9
AJ13199/pYAHN	27.9
AJ13199/pYEAS	29.7
AJ13199/pYFIK	28.4

Example 4. Effect of *yeaS*, *yahN*, and *yfiK* DNA fragments amplification on lysine production.

(1). As the lysine-producing bacterium belonging to the genus *Escherichia*, *E. coli* strain W3110 (TyrA) described in European Patent Publication No. 488424 to which plasmid pCABD2 was introduced, described in International Publication No. WO 95/16042) was used. Specifically, plasmid pCABD2, and each of the plasmid pMW118::*yahN*, pMW118::*yeaS*, pMW118::*yfiK* and pMW118 were introduced to *E. coli* strain W3110 (TyrA) to obtain the following strains:

W3110 (tyrA)/pCABD2+pMW118::*yahN*

W3110 (tyrA)/pCABD2+pMW118::*yeaS*

W3110 (tyrA)/pCABD2+pMW118::*yfik*

W3110 (tyrA)/pCABD2+pMW118.

Lysine productivity of these strains was estimated by culture. The composition of the used medium was as follows (g/l):

Glucose	40.0
$MgSO_4 \cdot 7H_2O$	1.0
$(NH_4)_2SO_4$	16.0
K_2HPO_4	1.0
$FeSO_4 \cdot 7H_2O$	0.01
$MnSO_4 \cdot 7H_2O$	0.01
Yeast extract (Difco)	2.0
Tyrosine	0.1

Adjusted to pH 7.0 and autoclaved at 115° C for 10 minutes. (Glucose and $MgSO_4 \cdot 7H_2O$ separately sterilized)

Pharmacopeial $CaCO_3$ 25 g/l (dry-heat-sterilized at 180°C for 2 h)

As antibiotics, 20 mg/l of streptomycin and 50 mg/l of ampicillin were added depending on a kind of a plasmid. Cultivation was conducted at 37°C for 30 hours with agitation at 115 rpm. The results are shown in Table 3.

Table 3

Strain	Lysine, g/l	Yield, (%)
W3110(tyrA)	0.08	0.2
W3110(tyrA)/pCABD2 + pMW118	12.2	30.5
W3110(tyrA)/pCABD2 + pMW118::*yahN*	13.8	34.5
W3110(tyrA)/pCABD2 + pMW118::*yeaS*	12.7	31.8
W3110(tyrA)/pCABD2 + pMW118::*yfiK*	12.2	30.5

The result in Table 3 shows that the produced amount and the yield based on consumed sugar of lysine is increased by enhancement of YahN and YeaS.

(2). As the lysine-producing bacterium belonging to the genus *Escherichia*, *E. coli* strain VL614 was used. This strain is a derivative of the known *E. coli* strain VL613 (SU Patent No. 1354458). In turn, the strain VL613 was obtained from the known strain Gif102 (Theze, J. and Saint Girons. J.Bacteriol., 118, 990-998, 1974) in the three steps:

At the first step the mutants resistant to 2 mg/ml S-(2-aminoethyl)-L-cysteine were selected and among them the strain VL611 was found capable to produce L-lysine.

At the second step the genes involved in sucrose utilization and located on the transposon Tn2555 (Doroshenko et al., Mol. Biologiya, 22, 645-658, 1988), were introduced into VL611 using phage P1-mediated transduction giving the strain VL612.

At the third step, the mutation *rhtA*23 from the strain VKPM B-3996, conferring resistance to threonine and homoserine (US Patent No. 5,175,107) was introduced into VL612 by phage P1 transduction giving the strain VL613.

The *E. coli* strain VL614 was obtained by transduction of the wild-type allele of the *rhtA* gene from the *E. coli* strain VKPM B-6204 (MG1655 *zbi*3058::Tn10) to VL613. Transductants were selected on L-medium containing 10 mg/l tetracyclin, and among them the strain VL614 ($rhtA^+$) sensitive to 10 g/l homoserine was found.

The strain VL614 was transformed with the pYGGA plasmid or with the pOK12 vector to obtain strains VL614/pYGGA (VKPM B-7719) and VL614/pOK12 (VKPM B-7722).

These strains were each cultivated at 37°C for 18 hours in a nutrient broth with 50 mg/l kanamycin, and 0.3 ml of the obtained culture was inoculated into 3 ml of a fermentation medium (Example 3) containing 0.3 g/l threonine, 0.3 g/l methionine and 50 mg/l kanamycin, in a 20 x 200 mm test tube, and cultivated at 37°C for 48 hours with a rotary shaker. After the cultivation, each accumulated amount of lysine and glutamate in the medium was determined by the known method.

The results are shown in Table 4.

Table 4

Strain	Lysine, g/l	Glutamate, g/l
VL614/pOK12	2.6	0.8
VL614/pYGGA	3.6	2.2

As shown in Table 4, the strain VL614/pYGGA accumulated lysine in a larger amount than the strain VL614/pOK12 in which the *yggA* gene was not enhanced. Besides, the strain VL614/pYGGA accumulated more glutamic acid than the strain VL614/pOK12.

Example 5. Effect of *yeaS*, *yahN*, and *yfiK* DNA fragments amplification on threonine, alanine, valine and isoleucine production.

As the threonine-producing bacterium belonging to the genus *Escherichia*, the *E. coli* strain VL2054 was used. This strain was derived from the known *E. coli* strain VKPM B-3996 (US Patent No. 5,175,107) as follows.

Initially, a new recipient strain was constructed in several steps:

- The plasmidless derivative of the strain VKPM B-3996 was selected after spontaneous elimination of pVIC40 plasmid.
- The wild-type allele of the *rhtA* gene from the *E. coli* strain VKPM B-6204 (MG1655 *zbi*3058::Tn10) was introduced into the thus obtained strain by phage P1 mediated transduction as in the Example 4.
- A mutation inactivating *kan* gene of the Tn5 transposon inserted into the *tdh* gene was obtained after NG mutagenesis and selection of kanamycin-sensitive cells still unable to degrade threonine. Thus the strain VL2053 was obtained.

On the other hand, the threonine operon from pVIC40 was cloned into integrative Mud vector under the P_R promoter of the phage lambda. In addition, the *cat* gene of Tn9 conferring the resistance to chloramphenicol was cloned into the same vector. The construct thus obtained was inserted into the chromosome of the *E. coli* strain C600 by use of the known method (US Patent No. 5,595,889) and transduced from

the thus obtained strain to VL2053, giving the new plasmidless threonine-producing strain VL2054. This strain accumulated in culture broth also alanine, valine and isoleucine.

The strain VL2054 was transformed with each of the plasmids pYEAS, pYFIK, and with the vector pUC21 to obtain *E. coli* strains VL2054/pYEAS (VKPM B-7707), VL2054/pYFIK (VKPM B-7712) and VL2054/pUC21 (VKPM B-7708).

These strains were each cultivated at 37°C for 18 hours in a nutrient broth with 100 mg/l ampicillin, and 0.3 ml of the obtained culture was inoculated into 3 ml of a fermentation medium (Example 3) containing 100 mg/l ampicillin, in a 20 x 200 mm test tube, and cultivated at 37°C for 48 hours with a rotary shaker. After the cultivation, each accumulated amount of threonine, alanine, valine and isoleucine in the medium was determined by known method.

The results are shown in Table 5.

As shown in Table 5, the strain VL2054/pYFIK accumulated threonine in a larger amount than the strain VL2054/pUC21 in which the *yfiK* gene was not enhanced. Besides, the strain VL2054/pYEAS accumulated more alanine, valine and isoleucine than the strain VL2054/pUC21 in which the *yeaS* gene was not enhanced.

Table 5

Strain	Amino acid accumulation, g/l			
	Threonine	Alanine	Valine	Isoleucine
VL2054/pUC21	5.8	0.4	0.31	0.15
VL2054/pYEAS	5.2	1.4	0.52	0.45
VL2054/pYFIK	8.8	0.5	0.22	0.14

Example 6. Effect of *yeaS* and *yfiK* DNA fragments amplification on histidine production.

As the histidine-producing bacterium belonging to the genus *Escherichia*, the strain *E. coli* VL2160 was used. This strain was obtained on the basis of the known strain NK5526 *hisG*::Tn10 (VKPM B-3384) by phage P1-mediated transduction of the $hisG^R$ mutation desensitizing ATP-phosphoribosyltransferase from the strain CC46 (Astvatsaturianz et al., Genetika, 24, 1928-1934, 1988). The strain *E. coli* VL2160 was transformed with each of the plasmids pYEAS, pYFIK, and with the vectors pUC21 to obtain *E. coli* strains VL2160/pYEAS (VKPM B-7753), *E. coli* VL2160/pYFIK (VKPM B-7754), *E. coli* VL2160/pUC21 (VKPM B-7752).

These strains were each cultivated at 37°C for 18 hours in a nutrient broth with 100 mg/l ampicillin, and 0.3 ml of the obtained culture was inoculated into 3 ml of the fermentation medium (Example 3) containing an increased amount of yeast extract (3 g/l) and 100 mg/l ampicillin, in a 20 x 200 mm test tube, and cultivated at 34°C for 68 hours with a rotary shaker.

After the cultivation, an accumulated amount of histidine in the medium was determined by known method. The results are shown in Table 6.

Table 6

Strain	Histidine, g/l
VL2160/pUC21	1.2
VL2160/pYEAS	1.8
VL2160/pYFIK	1.4

As shown in Table 6, the strains *E. coli* VL2160/ pYEAS and *E. coli* VL2160/pYFIK accumulated histidine in a larger amount than the strain *E. coli* VL2160/pUC21 in which the *yeaS* and *yfiK* genes were not enhanced.

Example 7. Effect of *yahN*, *yfiK* and *yeaS* DNA fragments amplification on proline production.

As the proline-producing bacterium belonging to the genus *Escherichia*, the strain VL2151 (W3350 *proB** Δ*putAP* Tn10) was used. This strain was obtained by transduction into W3350 of Δ*putAP* mutation linked to Tn10 and selecting tetracycline-resistant transductants unable to utilize proline as a sole carbon source. The thus obtained strain W3350 Δ*putAP* Tn10 was mutagenized with NG and mutants resistant to 20 mg/l of 3,4-dehydro-DL-proline were selected. Among them the strain VL2151 (W3350 *proB** Δ*putAP* Tn10) was found capable to produce proline.

The strain *E. coli* VL2151 was transformed with each of the plasmids pYEAS, pYFIK, pYAHN and with the vectors pUC21 to obtain *E. coli* strains VL2151/pYEAS (VKPM B-7714), VL2151/pYFIK (VKPM B-7713), VL2151/pYAHN (VKPM B-7748) and *E. coli* VL2151/pUC21 (VKPM B-7715).

These strains were each cultivated at 37°C for 18 hours in a nutrient broth with 100 mg/l ampicillin, and 0.3 ml of the obtained culture was inoculated into 3 ml of a fermentation medium (Example 3) containing 100 mg/l ampicillin, in a 20 x 200 mm test tube, and cultivated at 37°C for 48 hours with a rotary shaker. After the cultivation, an accumulated amount of proline in the medium was determined by known method. The results are shown in Table 7.

Table 7

Strain	Proline, g/l
VL2151/pUC21	1.8
VL2151/pYAHN	2.2
VL2151/pYEAS	2.1
VL2151/pYFIK	2.5

As shown in Table 7, the strains *E. coli* VL2151/pYFIK, *E. coli* VL2151/pYAHN and *E. coli* VL2151/pYEAS accumulated proline in a larger amount than the strain *E. coli* VL2151/pUC21 in which the *yfiK*, *yahN* and *yeaS* genes were not enhanced. The amplification of *yfiK* gene had the most pronounced effect.

Example 8. Effect of *yggA* DNA fragments amplification on arginine production.

As arginine-producing bacterium belonging to the genus *Escherichia*, the strain W3350 *argE*::Tn10/pKA10 was used. This strain harbors a plasmid, pKA10, containing DNA region from *Corynebacterium* (*Brevibacterium*) *flavum* which complements at least *argA* and *argE* mutations in the recipient strain of *E. coli* K-12 (Kharitonov A. and Tarasov A.P. Molecular Genetics, Microbiology and Virology. No.9, 29-33, 1986).

The strain *E. coli* W3350 *argE*::Tn10/pKA10 was transformed with the plasmid pYGGA, or with the vector pOK12 to obtain the strains *E. coli* W3350 *argE*::Tn10/pKA10, pYGGA (VKPM B-7716) and *E. coli* W3350 *argE*::Tn10/pKA10, pOK12 (VKPM B-7718).

The thus obtained transformants were each cultivated at 37°C for 18 hours in a nutrient broth with 100 mg/l ampicillin and 50 mg/l kanamycin, and 0.3 ml of the obtained culture was inoculated into 3 ml of a fermentation medium (Example 3) containing 100 mg/l ampicillin and 50 mg/l kanamycin, in a 20 x 200 mm test tube, and cultivated at 37°C for 48 hours with a rotary shaker. After the cultivation, an accumulated amount of arginine in the medium was determined by known method.

The results are shown in Table 8.

Table 8

Strain	Arginine, g/l
W3350 *argE*::Tn10/pKA10, pOK12	0.11
W3350 *argE*::Tn10/pKA10, pYGGA	0.46

As shown in Table 8, the strains *E. coli* W3350 *argE*::Tn10/pKA10, pYGGA accumulated arginine in a larger amount than the strain *E. coli* W3350 *argE*::Tn10/pKA10, pUC21 in which the *yggA* gene was not enhanced.

The following *E. coli* strains have been deposited (according to international deposition based on Budapest Treaty) in the Russian National Collection of Industrial Microorganisms (VKPM) on December 29, 1998 under the accession numbers shown in parenthesis.

AJ13199/pUC21 (VKPM B-7728)

AJ13199/pYAHN (VKPM B-7729)

AJ13199/pYEAS (VKPM B-7731)

AJ13199/pYFIK (VKPM B-7730)

VL614/pYGGA (VKPM B-7719)

VL614/pOK12 (VKPM B-7722)

VL2054/pYEAS (VKPM B-7707)

VL2054/pYFIK (VKPM B-7712)

VL2054/pUC21 (VKPM B-7708)

VL2160/pYEAS (VKPM B-7753)

VL2160/pYFIK (VKPM B-7754)

VL2160/pUC21 (VKPM B-7752)

VL2151/pYFIK (VKPM B-7713)

VL2151/pYEAS (VKPM B-7714)

VL2151/pYAHN (VKPM B-7748)

VL2151/pUC21 (VKPM B-7715)

W3350 *argE*::Tn10/pKA10, pYGGA (VKPM B-7716)

W3350 *argE*::Tn10/pKA10, pOK12 (VKPM B-7718)

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Leu Leu Trp Phe Ala Trp Cys Ser Met Arg Arg Gln Ser Thr Pro Gln
            100                 105                 110
Met Ser Thr Leu Gln Gln Pro Ile Ser Ala Pro Trp Tyr Val Phe Phe
        115                 120                 125
Arg Arg Gly Leu Ile Thr Asp Leu Ser Asn Pro Gln Thr Val Leu Phe
    130                 135                 140
Phe Ile Ser Ile Phe Ser Val Thr Leu Asn Ala Glu Thr Pro Thr Trp
145                 150                 155                 160
Ala Arg Leu Met Ala Trp Ala Gly Ile Val Leu Ala Ser Ile Ile Trp
                165                 170                 175
Arg Val Phe Leu Ser Gln Ala Phe Ser Leu Pro Ala Val Arg Arg Ala
            180                 185                 190
Tyr Gly Arg Met Gln Arg Val Ala Ser Arg Val Ile Gly Ala Ile Ile
        195                 200                 205
Gly Val Phe Ala Leu Arg Leu Ile Tyr Glu Gly Val Thr Gln Arg
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<222> (1)..(639)

<400> 11

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1 5 10 15

gcc att ttt att gtg ttg gtg cca ggg cca aat acc ctg ttt gta ctc 96
Ala Ile Phe Ile Val Leu Val Pro Gly Pro Asn Thr Leu Phe Val Leu
20 25 30

aaa aat agc gtc agt agc ggt atg aaa ggc ggt tat ctt gcg gcc tgc 144
Lys Asn Ser Val Ser Ser Gly Met Lys Gly Gly Tyr Leu Ala Ala Cys
35 40 45

ggt gta ttt att ggc gat gcg gta ttg atg ttt ctg gca tgg gct gga 192
Gly Val Phe Ile Gly Asp Ala Val Leu Met Phe Leu Ala Trp Ala Gly
50 55 60

gtg gcg aca tta att aag acc acc ccg ata tta ttc aac att gta cgt 240
Val Ala Thr Leu Ile Lys Thr Thr Pro Ile Leu Phe Asn Ile Val Arg
65 70 75 80

tat ctt ggt gcg ttt tat ttg ctc tat ctg ggg agt aaa att ctt tac 288
Tyr Leu Gly Ala Phe Tyr Leu Leu Tyr Leu Gly Ser Lys Ile Leu Tyr
85 90 95

gcg acc ctg aag ggt aaa aat agc gag gcc aaa tcc gat gag ccc caa 336
Ala Thr Leu Lys Gly Lys Asn Ser Glu Ala Lys Ser Asp Glu Pro Gln
100 105 110

tac ggt gct att ttt aaa cgc gcg tta att ttg agc ctg act aat ccg 384
Tyr Gly Ala Ile Phe Lys Arg Ala Leu Ile Leu Ser Leu Thr Asn Pro
115 120 125

aaa gcc att ttg ttc tat gtg tcg ttt ttc gta cag ttt atc gat gtt 432
Lys Ala Ile Leu Phe Tyr Val Ser Phe Phe Val Gln Phe Ile Asp Val
130 135 140

aat gcc cca cat acg gga att tca ttc ttt att ctg gcg gcg acg ctg 480
Asn Ala Pro His Thr Gly Ile Ser Phe Phe Ile Leu Ala Ala Thr Leu
145 150 155 160

gaa ctg gtg agt ttc tgc tat ttg agc ttc ctg att ata tct ggt gct 528
Glu Leu Val Ser Phe Cys Tyr Leu Ser Phe Leu Ile Ile Ser Gly Ala
165 170 175

ttt gtc acg cag tac ata cgt acc aaa aag aaa ctg gct aaa gtt ggc 576
Phe Val Thr Gln Tyr Ile Arg Thr Lys Lys Lys Leu Ala Lys Val Gly
180 185 190

aac tca ctg att ggt ttg atg ttc gtg ggt ttc gct gcc cga ctg gcg 624
Asn Ser Leu Ile Gly Leu Met Phe Val Gly Phe Ala Ala Arg Leu Ala
195 200 205

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Thr Leu Gln Ser
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Lys Asn Ser Val Ser Ser Gly Met Lys Gly Gly Tyr Leu Ala Ala Cys
35 40 45
Gly Val Phe Ile Gly Asp Ala Val Leu Met Phe Leu Ala Trp Ala Gly
50 55 60
Val Ala Thr Leu Ile Lys Thr Thr Pro Ile Leu Phe Asn Ile Val Arg
65 70 75 80
Tyr Leu Gly Ala Phe Tyr Leu Leu Tyr Leu Gly Ser Lys Ile Leu Tyr
85 90 95
Ala Thr Leu Lys Gly Lys Asn Ser Glu Ala Lys Ser Asp Glu Pro Gln
100 105 110
Tyr Gly Ala Ile Phe Lys Arg Ala Leu Ile Leu Ser Leu Thr Asn Pro
115 120 125
Lys Ala Ile Leu Phe Tyr Val Ser Phe Phe Val Gln Phe Ile Asp Val
130 135 140
Asn Ala Pro His Thr Gly Ile Ser Phe Phe Ile Leu Ala Ala Thr Leu
145 150 155 160
Glu Leu Val Ser Phe Cys Tyr Leu Ser Phe Leu Ile Ile Ser Gly Ala
165 170 175
Phe Val Thr Gln Tyr Ile Arg Thr Lys Lys Lys Leu Ala Lys Val Gly
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Thr Leu Gln Ser
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<222> (1)..(588)

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Ala Met Thr Pro Gly Pro Asn Asn Ile Leu Ala Leu Ser Ser Ala Thr
             20                  25                  30
tcg cat gga ttt cgt caa agt acc cgc gtg ctg gca ggg atg agt ctg   144
Ser His Gly Phe Arg Gln Ser Thr Arg Val Leu Ala Gly Met Ser Leu
         35                  40                  45
gga ttt ttg att gtg atg tta ctg tgt gcg ggc att tca ttt tca ctg   192
Gly Phe Leu Ile Val Met Leu Leu Cys Ala Gly Ile Ser Phe Ser Leu
     50                  55                  60
gca gtg att gac ccg gca gcg gta cac ctt ttg agt tgg gcg ggg gcg   240
Ala Val Ile Asp Pro Ala Ala Val His Leu Leu Ser Trp Ala Gly Ala
 65                  70                  75                  80
gca tat att gtc tgg ctg gcg tgg aaa atc gcc acc agc cca aca aag   288
Ala Tyr Ile Val Trp Leu Ala Trp Lys Ile Ala Thr Ser Pro Thr Lys
                 85                  90                  95
gaa gac gga ctt cag gca aaa cca atc agc ttt tgg gcc agc ttt gct   336
Glu Asp Gly Leu Gln Ala Lys Pro Ile Ser Phe Trp Ala Ser Phe Ala
            100                 105                 110
ttg cag ttt gtg aac gtc aaa atc att ttg tac ggt gtt acg gca ctg   384
Leu Gln Phe Val Asn Val Lys Ile Ile Leu Tyr Gly Val Thr Ala Leu
        115                 120                 125
tcg acg ttt gtt ctg ccg caa aca cag gcg tta agc tgg gta gtt ggc   432
Ser Thr Phe Val Leu Pro Gln Thr Gln Ala Leu Ser Trp Val Val Gly
    130                 135                 140
gtc agc gtt ttg ctg gcg atg att ggg acg ttt ggc aat gtg tgc tgg   480
Val Ser Val Leu Leu Ala Met Ile Gly Thr Phe Gly Asn Val Cys Trp
145                 150                 155                 160
gcg ctg gcg ggg cat ctg ttt cag cga ttg ttt cgc cag tat ggt cgc   528
Ala Leu Ala Gly His Leu Phe Gln Arg Leu Phe Arg Gln Tyr Gly Arg
                165                 170                 175
cag tta aat atc gtg ctt gcc ctg ttg ctg gtc tat tgc gcg gta cgc   576
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<400> 14

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Ser His Gly Phe Arg Gln Ser Thr Arg Val Leu Ala Gly Met Ser Leu
         35                  40                  45
Gly Phe Leu Ile Val Met Leu Leu Cys Ala Gly Ile Ser Phe Ser Leu
     50                  55                  60
Ala Val Ile Asp Pro Ala Ala Val His Leu Leu Ser Trp Ala Gly Ala
 65                  70                  75                  80
Ala Tyr Ile Val Trp Leu Ala Trp Lys Ile Ala Thr Ser Pro Thr Lys
                 85                  90                  95
Glu Asp Gly Leu Gln Ala Lys Pro Ile Ser Phe Trp Ala Ser Phe Ala
            100                 105                 110
Leu Gln Phe Val Asn Val Lys Ile Ile Leu Tyr Gly Val Thr Ala Leu
        115                 120                 125
Ser Thr Phe Val Leu Pro Gln Thr Gln Ala Leu Ser Trp Val Val Gly
    130                 135                 140
Val Ser Val Leu Leu Ala Met Ile Gly Thr Phe Gly Asn Val Cys Trp
145                 150                 155                 160
Ala Leu Ala Gly His Leu Phe Gln Arg Leu Phe Arg Gln Tyr Gly Arg
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Leu Pro Leu Gly Pro Gln Asn Ala Phe Val Met Asn Gln Gly Ile Arg
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cgt cag tac cac att atg att gcc tta ctt tgt gct atc agc gat ttg 144
Arg Gln Tyr His Ile Met Ile Ala Leu Leu Cys Ala Ile Ser Asp Leu
35 40 45
gtc ctg att tgc gcc ggg att ttt ggt ggc agc gcg tta ttg atg cag 192
Val Leu Ile Cys Ala Gly Ile Phe Gly Gly Ser Ala Leu Leu Met Gln
50 55 60
tcg ccg tgg ttg ctg gcg ctg gtc acc tgg ggc ggc gta gcc ttc ttg 240
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65 70 75 80
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Leu Trp Tyr Gly Phe Gly Ala Phe Lys Thr Ala Met Ser Ser Asn Ile
85 90 95
gag tta gcc agc gcc gaa gtc atg aag caa ggc aga tgg aaa att atc 336
Glu Leu Ala Ser Ala Glu Val Met Lys Gln Gly Arg Trp Lys Ile Ile
100 105 110
gcc acc atg ttg gca gtg acc tgg ctg aat ccg cat gtt tac ctg gat 384
Ala Thr Met Leu Ala Val Thr Trp Leu Asn Pro His Val Tyr Leu Asp
115 120 125
act ttt gtt gta ctg ggc agc ctt ggc ggg caa ctt gat gtg gaa cca 432
Thr Phe Val Val Leu Gly Ser Leu Gly Gly Gln Leu Asp Val Glu Pro
130 135 140
aaa cgc tgg ttt gca ctc ggg aca att agc gcc tct ttc ctg tgg ttc 480
Lys Arg Trp Phe Ala Leu Gly Thr Ile Ser Ala Ser Phe Leu Trp Phe
145 150 155 160
ttt ggt ctg gct ctt ctc gca gcc tgg ctg gca ccg cgt ctg cgc acg 528
Phe Gly Leu Ala Leu Leu Ala Ala Trp Leu Ala Pro Arg Leu Arg Thr
165 170 175
gca aaa gca cag cgc att atc aat ctg gtt gtg gga tgt gtt atg tgg 576
Ala Lys Ala Gln Arg Ile Ile Asn Leu Val Val Gly Cys Val Met Trp
180 185 190
ttt att gcc ttg cag ctg gcg aga gac ggt att gct cat gca caa gcc 624
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Arg Gln Tyr His Ile Met Ile Ala Leu Leu Cys Ala Ile Ser Asp Leu
35 40 45
Val Leu Ile Cys Ala Gly Ile Phe Gly Gly Ser Ala Leu Leu Met Gln
50 55 60
Ser Pro Trp Leu Leu Ala Leu Val Thr Trp Gly Gly Val Ala Phe Leu
65 70 75 80
Leu Trp Tyr Gly Phe Gly Ala Phe Lys Thr Ala Met Ser Ser Asn Ile
85 90 95
Glu Leu Ala Ser Ala Glu Val Met Lys Gln Gly Arg Trp Lys Ile Ile
100 105 110
Ala Thr Met Leu Ala Val Thr Trp Leu Asn Pro His Val Tyr Leu Asp
115 120 125
Thr Phe Val Val Leu Gly Ser Leu Gly Gly Gln Leu Asp Val Glu Pro
130 135 140
Lys Arg Trp Phe Ala Leu Gly Thr Ile Ser Ala Ser Phe Leu Trp Phe
145 150 155 160
Phe Gly Leu Ala Leu Leu Ala Ala Trp Leu Ala Pro Arg Leu Arg Thr
165 170 175
Ala Lys Ala Gln Arg Ile Ile Asn Leu Val Val Gly Cys Val Met Trp
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<220>
<223> Description of Artificial Sequence: primer for amplifying Escherichia coli yahN gene

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<210> 18
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<400> 18
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<220>
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<210> 20
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<213> Artificial Sequence

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<223> Description of Artificial Sequence: primer for amplifying Escherichia coli yeaS gene

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<223> Description of Artificial Sequence: primer for amplifying Escherichia coli yfiK gene

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<210> 22
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<223> Description of Artificial Sequence: primer for amplifying Escherichia coli yfiK gene

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<210> 23
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<223> Description of Artificial Sequence: primer for amplifying Escherichia coli yggA gene

<400> 23
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<210> 24
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<220>
<223> Description of Artificial Sequence: primer for amplifying Escherichia coli yggA gene

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What is claimed is:

1. A bacterium belonging to the genus *Escherichia* and having an ability to produce an L-amino acid, wherein the ability to produce the L-amino acid is increased by increasing an expression amount of at least one protein selected from the group consisting of the following proteins of (A) to (H):

(A) a protein having an amino acid sequence shown in SEQ ID NO: 10 in Sequence Listing;

(B) a protein which has an amino acid sequence including deletion, substitution, insertion, addition or inversion of one or several amino acids in the amino acid sequence shown in SEQ ID NO: 10 in Sequence Listing, and which has an activity of increasing the ability to produce the L-amino acid of the bacterium having the protein;

(C) a protein having an amino acid sequence shown in SEQ ID NO: 12 in Sequence Listing;

(D) a protein which has an amino acid sequence including deletion, substitution, insertion, addition or inversion of one or several amino acids in the amino acid sequence shown in SEQ ID NO: 12 in Sequence Listing, and which has an activity of increasing the ability to produce the L-amino acid of the bacterium having the protein;

(E) a protein having an amino acid sequence shown in SEQ ID NO: 14 in Sequence Listing;

(F) a protein which has an amino acid sequence including deletion, substitution, insertion, addition or inversion of one or several amino acids in the amino acid sequence shown in SEQ ID NO: 14 in Sequence Listing, and which has an activity of increasing the ability to produce the

L-amino acid of the bacterium having the protein;

(G) a protein having an amino acid sequence shown in SEQ ID NO: 16 in Sequence Listing; or

(H) a protein which has an amino acid sequence including deletion, substitution, insertion, addition or inversion of one or several amino acids in the amino acid sequence shown in SEQ ID NO: 16 in Sequence Listing, and which has an activity of increasing the ability to produce the L-amino acid of the bacterium having the protein.

2. The bacterium according to claim 1, wherein said L-amino acid is L-lysine and an expression amount of at least one protein selected from the group consisting of said proteins (A) to (D), (G) and (H) is increased.

3. The bacterium according to claim 1, wherein said L-amino acid is L-glutamic acid and an expression amount of at least one protein selected from the group consisting of said proteins (A) to (H) is increased.

4. The bacterium according to claim 1, wherein said L-amino acid is L-alanine and an expression amount of at least one protein selected from the group consisting of said proteins (C) and (D) is increased.

5. The bacterium according to claim 1, wherein said L-amino acid is L-valine and an expression amount of at least one protein selected from the group consisting of said proteins (C) and (D) is increased.

6. The bacterium according to claim 1, wherein said L-amino acid is L-histidine and an expression amount of at least one protein selected from the group consisting of said proteins (C) to (F) is increased.

7. The bacterium according to claim 1, wherein said L-

amino acid is L-proline and an expression amount of at least one protein selected from the group consisting of said proteins (A) to (F) is increased.

8. The bacterium according to claim 1, wherein said L-amino acid is L-threonine and an expression amount of at least one protein selected from the group consisting of said proteins (E) and (F) is increased.

9. The bacterium according to claim 1, wherein said L-amino acid is L-arginine and an expression amount of at least one protein selected from the group consisting of said proteins (G) and (H) is increased.

10. The bacterium according to claim 1, wherein said L-amino acid is L-isoleucine and an expression amount of at least one protein selected from the group consisting of said proteins (C) and (D) is increased.

11. The bacterium according to any one of claims 1 to 10, wherein a copy number of a DNA coding for said protein in a cell is increased.

12. The bacterium according to claim 11, wherein said DNA is carried on a multicopy vector in the cell.

13. The bacterium according to claim 11, wherein said DNA is carried on a transposon in the cell.

14. A method for producing an L-amino acid, comprising the steps of:

cultivating the bacterium as defined in claim 1 in a culture medium, to produce and accumulate the L-amino acid in the medium, and

recovering the L-amino acid from the medium.

15. The method according to claim 14, wherein said L-amino acid is L-lysine and said bacterium is one in which an

expression amount of at least one protein selected from the group consisting of said proteins (A) to (D), (G) and (H) is increased.

16. The method according to claim 14, wherein said L-amino acid is L-glutamic acid and said bacterium is one in which an expression amount of at least one protein selected from the group consisting of said proteins (A) to (H) is increased.

17. The method according to claim 14, wherein said L-amino acid is L-alanine and said bacterium is one in which an expression amount of at least one protein selected from the group consisting of said proteins (C) and (D) is increased.

18. The method according to claim 14, wherein said L-amino acid is L-valine and said bacterium is one in which an expression amount of at least one protein selected from the group consisting of said proteins (C) and (D) is increased.

19. The method according to claim 14, wherein said L-amino acid is L-histidine and said bacterium is one in which an expression amount of at least one protein selected from the group consisting of said proteins (C) to (F) is increased.

20. The method according to claim 14, wherein said L-amino acid is L-proline and said bacterium is one in which an expression amount of at least one protein selected from the group consisting of said proteins (A) to (F) is increased.

21. The method according to claim 14, wherein said L-amino acid is L-threonine and said bacterium is one in which an expression amount of at least one protein selected from the group consisting of said proteins (E) and (F) is increased.

22. The method according to claim 14, wherein said L-amino acid is L-arginine and said bacterium is one in which an

expression amount of at least one protein selected from the group consisting of said proteins (G) and (H) is increased.

23. The method according to claim 14, wherein said L-amino acid is L-isoleucine and said bacterium is one in which an expression amount of at least one protein selected from the group consisting of said proteins (C) and (D) is increased.

24. The method according to any one of claims 14 to 23, wherein a copy number of a DNA coding for said protein in a cell of said bacterium is increased.

25. The method according to claim 24, wherein said DNA is carried on a multicopy vector in the cell.

26. The method according to claim 24, wherein said DNA is carried on a transposon in the cell.

Abstract of the Disclosure

A bacterium belonging to the genus *Escherichia* and having an ability to produce an L-amino acid, wherein the ability to produce the L-amino acid is increased by increasing an expression amount of an L-amino acid excretion protein, and a method for producing the L-amino acid using the bacterium.

US

Declaration, Power Of Attorney and Petition

WE (I) the undersigned inventor(s), hereby declare(s) that:

My residence, post office address and citizenship are as stated below next to my name,

We (I) believe that we are (I am) the original, first, and joint (sole) inventor(s) of the subject matter which is claimed and for which a patent is sought on the invention entitled

METHOD FOR PRODUCING L-AMINO ACID

the specification of which

☒ is attached hereto.

☐ was filed on _____ as

Application Serial No. _____

and amended on _____ .

☐ was filed as PCT international application

Number _____

on _____ ,

and was amended under PCT Article 19

on _____ (if applicable).

We (I) hereby state that we (I) have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

We (I) acknowledge the duty to disclose information known to be material to the patentability of this application as defined in Section 1.56 of Title 37 Code of Federal Regulations.

We (I) hereby claim foreign priority benefits under 35 U.S.C. § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed. Prior Foreign Application(s)

Application No.	Country	Day/Month/Year	Priority Claimed
98124016	Russia	30/12/1998	☑ Yes ☐ No
99104431	Russia	09/03/1999	☑ Yes ☐ No
			☐ Yes ☐ No
			☐ Yes ☐ No

We (I) hereby claim the benefit under Title 35, United States Code, § 119(e) of any United States provisional application(s) listed below.

(Application Number)	(Filing Date)
(Application Number)	(Filing Date)

We (I) hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s), or § 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application.

Application Serial No.	Filing Date	Status (pending, patented, abandoned)

And we (I) hereby appoint: Norman F. Oblon, Registration Number 24,618; Marvin J. Spivak, Registration Number 24,913; C. Irvin McClelland, Registration Number 21,124; Gregory J. Maier, Registration Number 25,599; Arthur I. Neustadt, Registration Number 24,854; Richard D. Kelly, Registration Number 27,757; James D. Hamilton, Registration Number 28,421; Eckhard H. Kuesters, Registration Number 28,870; Robert T. Pous, Registration Number 29,099; Charles L. Gholz, Registration Number 26,395; Vincent J. Sunderdick, Registration Number 29,004; William E. Beaumont, Registration Number 30,996; Steven B. Kelber, Registration Number 30,073; Robert F. Gnuse, Registration Number 27,295; Jean-Paul Lavalleye, Registration Number 31,451; Timothy R. Schwartz, Registration Number 32,171; Stephen G. Baxter, Registration Number 32,884; Martin M. Zoltick, Registration Number 35,745; Robert W. Hahl, Registration Number 33,893; Richard L. Treanor, Registration Number 36,379; Steven P. Weihrouch, Registration Number 32,829; John T. Goolkasian, Registration Number 26,142; Marc R. Labgold, Registration Number 34,651; William J. Healey, Registration Number 36,160; and Richard L. Chinn, Registration Number 34,305; our (my) attorneys, with full powers of substitution and revocation, to prosecute this application and to transact all business in the Patent Office connected therewith; and we (I) hereby request that all correspondence regarding this application be sent to the firm of OBLON, SPIVAK, McCLELLAND, MAIER & NEUSTADT, P.C., whose Post Office Address is: Fourth Floor, 1755 Jefferson Davis Highway, Arlington, Virginia 22202.

We (I) declare that all statements made herein of our (my) own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Vitaliy Arkadievich LIVSHITS
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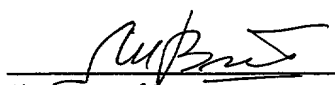
Citizen: Russia


Signature of Inventor

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Sumskoy proezd 5, Moscow, 113208,

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Date

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Date

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November 24, 1999
Date

Vladimir Veniaminovich ALESHIN
NAME OF FOURTH JOINT INVENTOR

Redsidence: Moscow Russia

Signature of Inventor

Citizen of: Russia

Post Office Address: pos Institute 6, Kaluga region, Borovsk, 249010, Russia

November 24, 1999
Date

Petr Vladimirovich TROSHIN
NAME OF FIFTH JOINT INVENTOR

Residence: Moscow Russia

Signature of Inventor

Citizen of: Russia

Post Office Address: kv. 129, Shipilovskiy pr. 67, Moscow, 115561, Russia

November 24, 1999
Date

Irina Lyvovna TOKHMAKOVA
NAME OF SIXTH JOINT INVENTOR

Residence: Moscow Russia

Signature of Inventor

Citizen of: Russia

Post Office Address: kv. 26, s/p Vysotka 1, Isrtrinskiy r-n, Moscow region, 143513, Russia

November 24, 1999
Date

NAME OF SEVENTH JOINT INVENTOR

Residence: _____

Signature of Inventor

Citizen of: _____

Post Office Address: _____

Date

NAME OF EIGHTH JOINT INVENTOR

Residence: _____

Signature of Inventor

Citizen of: _____

Post Office Address: _____

Date

NAME OF NINTH JOINT INVENTOR

Residence: _____

Signature of Inventor

Citizen of: _____

Post Office Address: _____

Date

Budapest treaty on the international recognition of the deposit of microorganisms for the purposes of patent procedure

To
State Scientific Centre of Russian Federation
GNIIGENETIKA

Moscow 113545
1-st Dorozhny proezd 1 Russia
name and address of depositor

INTERNATIONAL FORM
recept in the case of an original deposit issued pursuant to Rule 7.1 by the INTERNATIONAL DEPOSITARY AUTHORITY identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM

Identification reference given by the DEPOSITOR

Escherichia coli VL2054(pYEAS)

Accession number given by the INTERNATIONAL DEPOSITARY-AUTHORITY:
VKPM B- 7707

II.SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:

__x_ a scintific description Producer of threonine

____ a proposed taxonomic designation
(Mark with a cross where applicable)

III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the microorganism identified under I above,
1
which was received by it on 29.12.1998 (date of original deposit)

IV. RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I above was received by this International Depositary Authority
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V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: Russian National Collection of Industrial Microorganisms (VKPM)
Depositary
GNIIgenetika
Address: Russia 113545 Moscow
1 Dorozhny proezd 1

Signature (s) of person(s) having the power to represent the International Authority or authorized official(s):
Date: Sineoky S.P.

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I. IDENTIFICATION OF THE MICROORGANISM

Identification reference given by the DEPOSITOR	Accession number given by the INTERNATIONAL DEPOSITARY-AUTHORITY:
Escherichia coli VL2054 (pUC21)	VKPM B-7708

II.SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:

__x_ a scintific description Producer of threonine, alanine

____ a proposed taxonomic designation
(Mark with a cross where applicable)

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I. IDENTIFICATION OF THE MICROORGANISM

Identification reference given by the DEPOSITOR	Accession number given by the INTERNATIONAL DEPOSITARY-AUTHORITY:
Escherichia coli VL2054(pYFIK)	VKPM B- 7712

II.SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:

__x_ a scintific description Producer of threonine

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I. IDENTIFICATION OF THE MICROORGANISM

Identification reference given by the DEPOSITOR	Accession number given by the INTERNATIONAL DEPOSITARY-AUTHORITY:
Escherichia coli VL2151 (pYFIK)	VKPM B- 7713

II.SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:

__x_ a scintific description Producer of proline

____ a proposed taxonomic designation
(Mark with a cross where applicable)

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I. IDENTIFICATION OF THE MICROORGANISM

Identification reference given by the DEPOSITOR	Accession number given by the INTERNATIONAL DEPOSITARY-AUTHORITY:
Escherichia coli VL2151 (pYEAS)	VKPM B- 7714

II.SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:

__x_ a scintific description Producer of proline

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Identification reference given by the DEPOSITOR

Escherichia coli VL2151 (pUC21)

Accession number given by the INTERNATIONAL DEPOSITARY-AUTHORITY:
VKPM B- 7715

II.SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:

__x_ a scintific description Producer of proline

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I. IDENTIFICATION OF THE MICROORGANISM

Identification reference given by the DEPOSITOR	Accession number given by the INTERNATIONAL DEPOSITARY-AUTHORITY:
Escherichia coli W3350 argE::Tn10 (pKA10)(pYGGA)	VKPM B- 7716

II.SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:

__x_ a scintific description Producer of arginine

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Identification reference given by the DEPOSITOR	Accession number given by the INTERNATIONAL DEPOSITARY-AUTHORITY:
Escherichia coli W3350 argE::Tn10 (pKA10)(pOK12)	VKPM B- 7718

II.SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:

__x_ a scintific description Producer of arginine

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I. IDENTIFICATION OF THE MICROORGANISM

Identification reference given by the DEPOSITOR	Accession number given by the INTERNATIONAL DEPOSITARY-AUTHORITY:
Escherichia coli VL614 (pYGGA)	VKPM B-7719

II.SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:

__x_ a scintific description Producer of lysine

____ a proposed taxonomic designation
(Mark with a cross where applicable)

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Budapest treaty on the international recognition of the deposit of microorganisms for the purposes of patent procedure

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identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM

Identification reference given by the DEPOSITOR	Accession number given by the INTERNATIONAL DEPOSITARY-AUTHORITY:
Escherichia coli VL614 (pOK12)	VKPM B-7722

II.SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:

__x_ a scintific description Producer of lysine

____ a proposed taxonomic designation
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I. IDENTIFICATION OF THE MICROORGANISM

Identification reference given by the DEPOSITOR	Accession number given by the INTERNATIONAL DEPOSITARY-AUTHORITY:
Escherichia coli AJ13199(pUC21)	VKPM B- 7728

II.SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:

__x_ a scintific description Producer of glutamic acid

____ a proposed taxonomic designation
(Mark with a cross where applicable)

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Identification reference given by the DEPOSITOR	Accession number given by the INTERNATIONAL DEPOSITARY-AUTHORITY:
Escherichia coli AJ13199(pYAHN)	VKPM B- 7729

II.SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

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Identification reference given by the DEPOSITOR	Accession number given by the INTERNATIONAL DEPOSITARY-AUTHORITY:
Escherichia coli AJ13199(pYFIK)	VKPM B- 7730

II.SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

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Identification reference given by the DEPOSITOR	Accession number given by the INTERNATIONAL DEPOSITARY-AUTHORITY:
Escherichia coli AJ13199(pYEAS)	VKPM B- 7731

II.SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

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Escherichia coli VL2151 (pYAHN)	VKPM B- 7748

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Budapest treaty on the international recognition of the deposit of microorganisms for the purposes of patent procedure

To
State Scientific Centre of Russian Federation
GNIIGENETIKA
.
Moscow 113545
!-st Dorozhny proezd 1 Russia
name and address of depositor

INTERNATIONAL FORM
recept in the case of an original deposit issued pursuant to Rule 7.1 by the INTERNATIONAL DEPOSITARY AUTHORITY identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM

Identification reference given by the DEPOSITOR	Accession number given by the INTERNATIONAL DEPOSITARY- AUTHORITY:
Escherichia coli VL2160 (pUC21)	VKPM B- 7752

II.SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:

__x_ a scintific description Producer of histidine

____ a proposed taxonomic designation
(Mark with a cross where applicable)

III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the microorganism identified under I above,
1
which was received by it on 29.12.1998 (date of original deposit)

IV. RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I above was received by this International Depositary Authority
on (date of original deposit) and a request to convert the
original deposot to a deposit under the Budapest Treaty was received by it on
(date of receipt of request for conversion)

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: Russian National Collection of Industrial Microorganisms (VKPM)
GNIIgenetika
Address: Russia 113545 Moscow
1 Dorozhny proezd 1

Signature (s) of person(s) having the power to represent the International Depositary Authority or authorized official(s):
Date: Sineoky S.P.

1 Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired

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I. IDENTIFICATION OF THE MICROORGANISM

Identification reference given by the DEPOSITOR	Accession number given by the INTERNATIONAL DEPOSITARY-AUTHORITY:
Escherichia coli VL2160 (pYEAS)	VKPM B- 7753

II.SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

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Depositary
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Address: Russia 113545 Moscow
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I. IDENTIFICATION OF THE MICROORGANISM

Identification reference given by the DEPOSITOR	Accession number given by the INTERNATIONAL DEPOSITARY-AUTHORITY:
Escherichia coli VL2160 (pYFIK)	VKPM B- 7754

II.SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:

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